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DEVELOPMENT OF IMMUNODIAGNOSTIC TOOLS FOR STUDYING THE ETIOLOGY AND EPIDEMIOLOGY OF GREEN TURTLE FIBROPAPILLOMATOSIS

Paul A. Klein, Ph.D.,[1,2,3] Larry Herbst, D.V.M., M.S.,[5,7] Elliott Jacobson, D.V.M., Ph.D.,[3,4] Karen Anne Bjorndal, Ph.D.,[3,6] Alan B. Bolten, Ph.D.,[3,6] Bobby R. Collins, D.V.M.,[5] and Ellis C. Greiner, Ph.D.[7]

Honolulu Laboratory
Southwest Fisheries Science Center
National Marine Fisheries Service, NOAA
2570 Dole Street, Honolulu, Hawaii 96822-2396

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[1]Department of Pathology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida 32611

[2]Interdisciplinary Center For Biotechnology Research, University of Florida, Gainesville, Florida 32611

[3]Program in Biotechnologies for the Ecological, Evolutionary and Conservation Sciences (BEECS), University of Florida, Gainesville, Florida 32611

[4]Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611

[5]Division of Animal Resources, University of Florida, Gainesville, Florida 32611

[6]Archie Carr Center For Sea Turtle Research, University of Florida, Gainesville, Florida 32611

[7]Department of Infectious Diseases, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611

PREFACE

This report provides the results of research seeking to develop diagnotic tests to identify antigens that can be used to monitor populations of green turtles exposed to the agent responsible for fibropapilloma-type tumors. The work was conducted, in part, with funds supplied by the Southwest Fisheries Science Center Honolulu Laboratory's research program on threatened and endangered marine turtles.

The incidence of life-threatening tumors on green turtles in the Hawaiian Islands has increased to epidemic proportions during recent years. A similar situation exists among green turtles at certain sites in Florida, the Caribbean, and other locations worldwide. The cause of this disease, known as fibropapillomatosis, remains unknown. Death appears to be the usual result of the disease and, therefore, the impact on the afflicted populations may have serious consequences. The disease represents one more threat to the survival of green turtles worldwide. The nature of this disease and its cause must be determined in order to develop a long-term management program. The findings presented in the present report exemplify progress in this direction which must be followed up with additional research.

Because this report was prepared by independent investigators, its statements, findings, conclusions, and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA.

George H. Balazs
Zoologist
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ABSTRACT

The objective of this research is to apply the principles of immunology and seroepidemiology to the study of epidemic fibropapillomatosis (GTFP) in the green turtle, *Chelonia mydas*. Initial work has progressed on a broad front to develop the analytical and diagnostic tools necessary to study this disease. Because monoclonal antibodies (mabs) specific for immunoglobulin (Ig) are critical reagents in a variety of diagnostic immunoassays, green turtle immunoglobulins were purified from pooled plasma and a battery of mabs against turtle Ig light chain and 3 Ig heavy chain classes were produced. Validation of these mab reagents has been initiated by studying their ability to detect rising antibody titers in turtles inoculated with specific antigens. Toward the goal of identifying the effector cells responsible for host resistance to or recovery from fibropapillomatosis, green turtle blood cell morphology and cytochemical staining properties were characterized and development of mabs against specific leukocyte types was begun. Twenty-four mabs against turtle blood cells are in various stages of development. Characterization of one mab which is specific for green turtle heterophils is nearly complete. In a preliminary effort to identify GTFP-specific antigens for use in developing immunodiagnostic tests, mice were immunized with GTFP and the production of anti-GTFP mabs begun. Eight mabs of unknown specificity derived from a single fusion experiment have been retained for evaluation of their GTFP specificity. Also, preliminary tests of green turtle sera for anti-GTFP antibodies were conducted on crude tumor preparations using the newly produced anti-turtle Ig mabs. The initial results have been inconclusive and more definitive experiments await the development of more highly purified GTFP antigen preparations. Finally, to discover the etiology and understand the pathogenesis of GTFP a series of tumor transmission experiments were undertaken. Nine one-year-old, captive-reared green sea turtles were exposed by various routes to GTFP or possible etiologic agents. During the 12-month observation period only the two turtles inoculated with fibropapilloma developed tumors and only at the site where they received twice freeze-thawed GTFP homogenate. Further experiments are underway to differentiate between tumor cell transplantation and virus transmission. Overall, a substantial amount of progress has been made in the first 8 months of the project covered by this report. Work must continue to identify the etiologic agent and relevant GTFP antigens so that immunodiagnostic assays can be developed for use in epidemiologic surveys of green turtle populations.

INTRODUCTION

Green turtle fibropapillomatosis (GTFP) was first described in 1938 in green turtles, *Chelonia mydas*, from the Florida Keys (Lucke, 1938; Smith and Coates, 1938). In the last decade the prevalence of this disease has increased significantly and it has become a growing threat to green turtle populations worldwide (Balazs and Pooley, 1991). In addition, this or a similar disease may be affecting olive ridley, *Lepidochelys olivacea* (A. Chaves et al.; P. Plotkin, pers. comm.) and loggerhead, *Caretta caretta* populations (J. Harshbarger, pers. comm.). Comparative pathologic studies suggest that green turtle fibropapillomatosis may be caused by an oncogenic virus such as papillomavirus (Sundberg, 1987), herpesvirus (Raynaud and Adrian, 1976; Jacobson et al., 1986; Hedrick et al., 1990), poxvirus (Hirth et al., 1969; Pulley and Shively, 1973; O'Connor et al., 1980), or retrovirus (Francis-Floyd et al., 1993). With the exception of a herpesvirus described by Jacobson et al. (1991) however, viral inclusions or viral particles have not been observed in tumors. Preliminary screening of tumors using mammalian and avian papillomavirus DNA probes have failed to demonstrate the presence of homologous papillomavirus DNA (Jacobson et al., 1989). Although several pathogens and parasites have been associated with tumors, the etiology of GTFP remains unknown.

Without an identified pathogen to isolate and characterize, fulfillment of Koch's postulates for this disease may be years away. Meanwhile, it is critical to our understanding of this disease's impact on worldwide green turtle populations to have practical diagnostic methods to assess the level of exposure of various turtle populations to this disease. Identification of turtles with gross clinical signs of the disease (evidence of tumor growth on the external body surfaces) does not reveal the true extent of the disease in the population. Many turtles may have been infected with the causative agent, e.g., virus, but have not yet developed overt clinical disease (visible tumor growth). Such subclinically infected turtles may be carriers of the pathogen and spread it to healthy animals. Furthermore, we do not know whether the disease is uniformly fatal, or whether some turtles may actually recover and develop immunity. We cannot detect these individuals at the present time and the principal thrust of this research will be to develop specific and practical serological immunoassays with which to monitor green turtle populations for GTFP.

In addition, even after an etiologic agent has been identified and diagnostic tests developed, it will be critical to long-term management of sea turtle stocks to understand the factors which have rendered apparently healthy turtles more susceptible to this disease and allowed GTFP to become pandemic. This cannot be accomplished without concurrent studies aimed at improving our limited understanding of the function of the green turtle's immune system. In the case of fibropapillomatosis in the green sea turtle, failure of the turtle's immune system to

either recognize or to eliminate the relevant pathogen(s) and/or early papilloma tumor cells may be a major factor in the pathogenesis of this disease. Many factors may suppress the immune system and impair its ability to cope successfully with disease-producing agents. These include poor nutrition, toxins, disease, stress, and temperature. Any one or a combination of these factors can contribute to the loss of immunological competence and lead to the development or persistence of fibropapilloma disease (see McMichael, 1967; Duncan et al., 1975; Chretien et al., 1978 for other species). Therefore, an essential part of this research will be to further characterize the immune system of the green sea turtle and to evaluate the performance of both cell-mediated and antibody-mediated defense mechanisms in coping with disease challenge in an effort to understand the factors which render sea turtles susceptible to this disease.

Overall, these efforts will allow us to critically measure the performance of the sea turtle's immune system under a variety of conditions. Finally, and most important, immunoassays will be developed into reliable and practical immunodiagnostic tests with which to identify and monitor populations for GTFP.

The specific research goals for this year were as follows:

(1) Isolate and purify green turtle immunoglobulins and begin production of monoclonal and polyclonal antibodies to turtle immunoglobulins.

(2) Characterize green turtle blood cells by cytology and flow cytometry and begin production of monoclonal antibodies to specific turtle blood cell types.

(3) Evaluate methods to prepare fibropapilloma cell antigens for immunoassay and develop specific antigen preparations from putative etiologic agents.

(4) Conduct transmission studies to demonstrate an infectious etiology for fibropapillomatosis.

MATERIALS AND METHODS

Immunoglobulin Isolation

Globulins from a 50 ml sample of plasma from an individual green turtle, *C. mydas* and from a 100 ml pooled sample from three green turtles from Marathon, Florida were precipitated with saturated ammonium sulphate (33% v/v). The precipitate was resuspended in phosphate buffered saline (pH 7.4) + 0.02% azide (PBS-azide) and the precipitation repeated. The precipitate was dialyzed into either PBS-azide or 0.01 M Tris buffer pH 8.0 and adjusted to a final protein concentration of 2 mg/ml.

One portion (5 ml) of the globulin rich preparation in Tris buffer was applied to a diethyl aminoethyl (DEAE) anion exchange column and eluted in steps with 0.01 M Tris buffer containing either 0.125 M NaCl, 0.25 M NaCl, 0.5 M NaCl, or 1.0 M NaCl. Another portion (18 ml) of the globulin rich preparation in PBS-azide was also applied to a 2.5 x 100cm Sephacryl S-300 column in order to separate proteins on the basis of size. Fractions were eluted with PBS-azide at a 30 ml/hr flow rate and collected using a Gilson fraction collector. Selected eluted protein fractions were reduced by boiling for 5 minutes in Laemmli sample buffer (Laemmli, 1970) with 2-mercaptoethanol, and examined by SDS polyacrilamide gel electrophoresis (SDS-PAGE) using a Pharmacia Phastgel apparatus. Fractions containing similar protein composition were pooled and concentrated in Amicon Centriprep^{R}-10 concentrators (W. R. Grace & Co., Beverly, MA 01923 U.S.A.).

An affinity column was prepared using monoclonal antibody HL 673 which is specific for the immunoglobulin light chain of desert tortoise (Schumacher et al., *in press*) covalently linked to a Affi-prep^{R} Hz hydrazide support gel (Bio-Rad Laboratories, Richmond, CA 94804 U.S.A.). This monoclonal antibody was found to cross-react with putative light chain of the green turtle in enzyme-linked immunosorbent assays (ELISA) and western blotting. Two mg of purified mab HL 673 was dialyzed into oxidation buffer (0.02 M Sodium Acetate, 0.15 M Na Cl, pH 5.0) to a concentration of 2 mg/ml and reacted with 20 ul of Sodium Periodate stock solution (0.5 M $NaIO_4$) in a foil covered reaction vessel for 45 minutes at room temperature. The oxidation reaction was stopped by addition of 5 ul glycerol. The oxidized antibody was dialyzed into coupling buffer (0.1 M Sodium Acetate, 1.0 M NaCl, pH 4.5) overnight and then incubated with approximately 2 ml of settled Affi-prep^{R} Hz support beads overnight at room temperature. The antibody coupled beads were then washed with 0.5 M NaCl, 0.01 M phosphate buffer, pH 7.5 and stored at 4°C. The column was prepared, conditioned with elution buffer (0.1 M glycine, pH 2.7), washed with PBS-azide, and then 1 ml (2 mg) of green turtle globulin preparation was applied. After washing the column with 10 volumes of PBS-azide the bound protein was eluted with 0.1 M glycine, pH 2.7. Fractions (1 ml) were collected and neutralized with 45 ul of 1.0 M Tris, 0.01% azide). Eluted proteins were concentrated and examined with SDS-PAGE.

Characterization of Turtle Blood Cells

Peripheral blood was collected from the dorsal cervical sinus of six green turtles into lithium heparin vacutainer tubes. Six to ten blood smears were prepared from each sample and air dried. One blood film was stained with Wright-Geimsa and examined to evaluate blood cell morphology. The remaining slides were subjected to a battery of cytochemical stains: Periodic Acid-Schiff's (PAS), Toluidine Blue (TB), Benzidine Peroxidase

(BP), Chloroacetate Esterase (CAE), Acid Phosphatase, and alpha-Naphthylbutyrate Esterase (NBE). Staining patterns within individual cells were noted.

Antigen Preparation for Immunization and Screening

Normal Tissue Homogenate

Normal tissues were collected aseptically from a turtle that was euthanized due to severe fibropapillomatosis. A homogenate containing equal volumes of plasma, washed erythrocytes, muscle, kidney, and liver was prepared by freeze fracturing and grinding in ice-cold sterile PBS. The preparation was coarse filtered through syringe needles until the material could pass through a 23-gauge needle. Protein concentration was measured using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL 61105 U.S.A.). Aliquots were stored at -20°C until needed.

Blood Cell Preparation

Peripheral blood leukocytes were prepared from several green turtle blood samples using Ficoll-Sodium Diatrizoate (density 1.077-1.080 g/ml) Lymphocyte Separation Medium (LSM^R, Organon Teknika, Durham, NC). The resulting "buffy coat" was enriched for monocytes and lymphocytes but thrombocytes and granulocytes remained plentiful. Aliquots of fresh cells were prepared for each inoculation.

Fibropapilloma Homogenate

Fibropapilloma tissue for inoculation was collected aseptically and prepared by freeze fracturing and grinding in ice cold sterile PBS. The preparation was coarse filtered through syringe needles until the material could pass through a 20-gauge needle. Protein concentration was measured as described above and aliquots were stored at -20°C until needed.

Extracts of fibropapilloma and normal skin for use as crude antigens in screening immunoassays were prepared by grinding minced tissue in a homogenizer (Tekmar^R Tissumizer, Tekmar, Cincinnati, OH 45222 U.S.A.) or glass tissue grinder in ice cold sterile PBS/azide plus 0.2 mM PMSF and 1 mM EDTA. To prepare aqueous extract the crude homogenate was centrifuged (10,000 rpm for 15 minutes at 4°C), and the supernatant was filtered through a 0.4 u filter. Aliquots were stored at -20°C until needed.

Membrane bound proteins were extracted from the crude homogenate pellet with ice cold 0.05 M tris-HCl, pH 7.4 containing 5 mM EDTA and 0.5% (v/v) precondensed Triton X-114 (Sigma, St. Louis, MO 63178) (Bordier, 1981). The resulting detergent phase was enriched for proteins with hydrophobic

domains. Aliquots were stored at -20°C until needed.

Mouse Immunization Protocols

Anti-turtle Immunoglobulin Immunizations

One female Balb/c mouse was immunized with 6 ug of affinity purified turtle immunoglobulin in Ribi's adjuvant subcutaneously. Booster immunizations were repeated in 2 and 4 weeks. The final booster was 17 ug of antigen intraperitoneally. Fusion was performed 4 days after the last inoculation.

Two female Balb/c mice 6-8 weeks old were immunized with a combination (50 ug total protein) of 5.7S and 7S-rich green turtle immunoglobulins from the DEAE column (see fig. 1 and Ig isolation results below) in Ribi's adjuvant (Ribi Immunochem Research, Hamilton, MT 59840 U.S.A.) at several subcutaneous sites. Booster immunizations were performed at 2 and 4 weeks (50 ug antigen per mouse). Immunizations were continued at 2- to 4-week intervals for a total of seven immunizations. These final boosts included IgM-rich fraction with approximately 100 ug total protein.

Anti-GTFP and Leukocyte Immunizations

Production of monoclonal antibodies against fibropapilloma tissue and against leukocyte surface antigens involved a two-stage immunization protocol designed to improve the chances of recovering specific antibodies of interest (Matthew and Sandrock, 1987; Williams, et al., 1992). First, mice were given a series of inoculations accompanied by immunosuppressive therapy designed to deplete the immune system of lymphocytes reacting to normal turtle antigens. This is followed by inoculation with tissue containing the antigens of interest.

Six female Balb/c mice were given intraperitoneal injections of 0.1 ml (approx. 500 ug protein) of normal tissue homogenate (described above). This was followed in 10 minutes by an intraperitoneal injection of cyclophosphamide (Sigma, St. Louis, MO 63178 U.S.A.) 100mg/kg. The cyclophosphamide treatment was repeated at 24 and 48 hours. This tolerization procedure was repeated every 2 weeks for a total of four treatments. Following a 2-week rest period, the mice were inoculated with 0.1 ml (approx. 300 ug protein) tumor antigen preparation or turtle leukocytes (approx. 5×10^6 cells) in Ribi's adjuvant. These immunizations were repeated in 2 weeks and again, 2 days later. Four days after the final inoculation, the mice were euthanized. Monoclonal antibody production followed the standard protocol (below).

Monoclonal Antibody (Mab) Production

Four days following the final booster immunization, mice were euthanized under methoxyflurane anesthesia and their spleens removed. Splenocytes were prepared by mechanical disaggregation washed and fused with log phase SP2/0 mouse myeloma cells in a 7:1 ratio using 50% polyethylene glycol 1500 media (PEG) (Boeringer Mannheim, Germany). Approximately 9 x 10^7 splenocytes were fused each time in 1 ml of PEG plus 30 ml DMEA. After pelleting, cells were resuspended in fusion media (DME-PC, 1 x HAT, 25% Conditioned Media, 20% Horse Serum). Cells were seeded into 96 well culture plates, and wells were monitored for growth of hybridomas.

Screening was begun on growth-positive wells 10-14 days post fusion. The supernatant was removed and tested for antibody reactivity against specific antigens (below). Hybridoma mass cultures of interest were transferred to 24 well plates and maintained. These mass cultures were retested in about 7 days. Mass cultures of interest were frozen and selected cultures were cloned by limiting dilution.

Anti-green Turtle Immunoglobulin Mabs

Hybridoma mass culture supernatants were screened against each of the three turtle immunoglobulin rich pools using ELISA. Secondary screening was done by western blotting. Validation of putative antiturtle immunoglobulin mabs involved using them to detect antigen specific antibodies in plasma samples from specifically immunized green turtles.

ELISA procedure.--Each well of a microtiter plate (Maxisorp F96, NUNC, Kamstrup, Denmark) was coated with 50 µl of antigen at a concentration of 10 µg/ml in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.02% NaN_3 (PBS-azide) and incubated at 4°C overnight. The wells were washed four times with PBS-azide containing 0.05% tween-20 (PBS-tween) by an automatic ELISA washer (EAW II, LT-Labinstruments, 5082 Groedig/ Salzburg, Austria) and then blocked with 250 µl/well of PBS-azide containing 1% bovine serum albumin at room temperature for 60 minutes or at 4°C overnight. After four more washes, 50 µl of mass culture supernatant was added to individual wells and incubated at room temperature for 60 minutes. The wells were washed again as described and 50 µl of a 1:1000 dilution of alkaline phosphatase conjugated rabbit antimouse IgG whole molecule (Sigma, St. Louis, MO 63178, U.S.A.) was added to each well. The wells were incubated at room temperature for 60 minutes and washed as described. One-hundred µl of p-nitrophenyl phosphate disodium (Sigma, St. Louis, MO 63178, U.S.A.) (1 mg/ml prepared in 0.01 M sodium bicarbonate buffer, pH 9.6 containing 2 mM $MgCl_2$) was added to each well and

incubated in the dark at room temperature for 60 minutes. Optical density of each well at a wavelength of 405 nm was measured in an ELISA plate reader (EAR 400 AT, SLT-Labinstruments, 5082 Groedig/Salzburg, Austria). Positive and negative controls, included on each plate, consisted of replacing mass culture supernatant with immune mouse serum and cell culture medium, respectively.

Western blotting.--To help demonstrate the specificity of our monoclonal antibodies, 150 ug of green turtle globulins were separated by SDS-PAGE under reducing conditions, using a precast 10% Tris-Glycine gel (NOVEX, San Diego, CA 92121, U.S.A.) as previously described (Laemmli, 1970). The proteins were then electrophoretically transferred from the gel to a nitrocellulose sheet (NC, BA-S 83, Schleicher & Schuell, Inc., Keene, NH 03431, U.S.A.) (Harlow and Lane, 1988; Towbin et al., 1979), using the Novex "Western Transfer Apparatus" following the instructions provided by Novex. A Tris-Glycine buffer, pH 8.3, (Novex) in 20% methanol was used as transfer buffer. Blotting time was 120 minutes at 30 volts. Once the transfer was complete, the nitrocellulose was blocked immediately with PBS-azide containing 5% nonfat dry milk and incubated at room temperature on a rocker overnight. The nitrocellulose was then washed three times (5 minutes per wash) with PBS-tween and placed into a trough-manifold (PR 150 Mini Deca Probe, Hoeffer Scientific Instruments, San Francisco, CA 94107, U.S.A.). Three-hundred μl of mass culture supernatant were loaded per channel and incubated on the nitrocellulose for 90 minutes at room temperature on a rocker. The nitrocellulose membrane was washed three more times and then incubated with 300 ul of a 1:1000 dilution of alkaline phosphatase conjugated rabbit antimouse IgG whole molecule (Sigma, St. Louis, MO 63178 U.S.A.) for 90 minutes at room temperature. The membrane was then removed from the manifold, washed three times and developed with substrate buffer (0.1M Tris HCl, 1 mM $MgCl_2$, pH 8.8) containing 44 μl of nitroblue tetrazolium chloride (NBT) and 33 μl of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) per 10 ml of substrate buffer. NBT and BCIP were commercially obtained (Immunoselect™, Gibco BRL, Gaithersburg, MD 20877, U.S.A.).

Validation experiments.--The specificity of putative antiturtle immunoglobulin mabs were validated by their ability to detect an increasing anti-DNP antibody response in turtles immunized with DNP-BSA. Two captive-reared green turtles maintained at Cayman Turtle Farm, Grand Cayman, British West Indies were inoculated biweekly with 250 ug DNP-BSA in Ribi's adjuvant for a total of six inoculations. A pre-inoculation blood sample and biweekly test bleedings were collected between inoculations. Serial two-fold dilutions of plasma samples were tested by ELISA as described in methods above but using 50 ul DNP-BSA (1.25 ug/ml) coated per well as antigen, 0.1 M glycine for blocking, and 50 ul of test monoclonal antibody cloning supernatant as first

antibody. A competitive inhibition ELISA was used to verify that the turtle plasma proteins detected by each mab were DNP-specific. Plasma samples with peak antibody titers to DNP-BSA from the two immunized turtles (1:40 dilution) were incubated at 4°C overnight with increasing concentrations of soluble hapten (DNP-glycine in PBS-azide). These plasma samples were then used in an ELISA as described above.

Anti-GTFP Mabs

Screening of hybridoma mass culture supernatants was multi-tiered. Fusion supernatants were first screened against green turtle immunoglobulins by ELISA. ELISA negative mass cultures were retained and screened against aqueous (1 mg/ml) and TX-114 (1:20 dilution) extracts of tumor via immunoblot. Immunoblots were performed by incubating each antigen preparation (50 ul per well) on nitrocellulose membranes overnight at 4°C using a minifold^{R} blotting apparatus (Schleicher and Schuell, Inc., Keene, NH 03431 U.S.A.). The membranes were then washed and processed as for western blotting (above) using hybridoma mass culture supernatants as primary antibody. Immunoblot positive hybridomas were saved and screened by western blotting against the same antigen preparations.

Anti-leukocyte Mabs

Fusion mass culture supernatants were first screened against green turtle immunoglobulins by ELISA. ELISA negative hybridomas were retained and screened against peripheral blood leukocyte preparations using flow cytometry and a magnetic bead rosetting assay.

Flow cytometry.--Turtle leukocytes were placed in 96-well Falcon plates in aliquots of 1 million cells per well and incubated with various hybridoma supernatants for 30 minutes on ice. Controls were incubated with cell culture media or with an antihuman HLA mab (W6/32). The cells were washed three times by pelleting (1500 rpm for 3 minutes) and resuspension in PBS with 0.1% azide. Cells were then incubated on ice with FITC labelled Fab' fragment of sheep anti-mouse Ig (Sigma) in PBS with 0.1% azide and 1% BSA for 30 minutes. After washing, the stained cells were analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA 95131 U.S.A.) for light scatter properties and fluorescence (Shapiro, 1988). Mabs of interest were selected based on increased fluorescence of certain leukocyte subpopulations, compared to controls.

Magnetic bead rosetting assay.--Magnetic beads coated with sheep anti-mouse Ig Dynabeads M280 (Dynal A.S., N-0212 Oslo, Norway) were incubated with hybridoma supernatants for 24 hours at 4°C to sensitize them. After washing three times in PBS-azide the beads were resuspended in 1% BSA in PBS-azide at a concentration of

5 x 10^6 beads per ml. Approximately 1 x 10^5 turtle leukocytes per well were incubated with 2.5 x 10^5 sensitized beads in 100 ul total volume 1% BSA for 30 minutes in 24 well plates (Costar, Cambridge, MA 02140 U.S.A.) at 4°C on a rocker. Each well was examined under light microscope and scored for binding of beads to turtle cells. To identify the cell type(s) reacting with the beads rosetted cells were enriched by holding the plate to a magnet through repeated washes with PBS. The rosetted cells were then settled onto glass slides, stained, and examined for morphology.

Screening for Green Turtle Antibodies Against GTFP

Aqueous and TX-114 fibropapilloma preparations were separated by SDS-PAGE and blotted onto nitrocellulose paper. Western blots were performed using diluted plasma from eight GTFP-affected and five GTFP-free turtles as primary antibody. After washing, the membrane was then incubated with newly developed monoclonal mouse antiturtle Ig light chain. The membrane was then washed and incubated with alkaline phosphatase labelled rabbit antimouse IG followed by washing and incubation in substrate, as described in detail above (see western blotting).

Transmission Study

The experiments described here were begun in December 1991 and were designed to test several hypotheses about the etiology of this disease and its mode of transmission. In addition, experimentally transmitted fibropapillomas would provide a source of antigen for diagnostic test development.

Nine yearling green turtles that had been hatched and reared in captivity under quarantine in artificial seawater (Sea World, Orlando, FL) were used for the 1991 transmission experiments.

They were transported to holding tanks at the Hidden Harbor Marine Environmental Project, Marathon, FL and maintained in filtered natural sea water. Turtles were assigned to one of four treatment groups. Three turtles (group A) were housed with several larger tumor-bearing turtles to see if contact and water exposure was an effective route of exposure to the disease. Three turtles (group B) were given subcutaneous injections of ova from the blood fluke *Learedius learedii* (Spirorchidae) or an, as yet, unidentified trematode species to test the hypothesis that these eggs may play a role in inducing tumor growth. The ova were harvested from the spleens of fresh dead turtles and washed in sterile saline. Approximately 50 viable eggs suspended in 0.1 ml of sterile saline were injected at sites in the upper

eyelid, foreflipper, and rear flipper. *Learedius* ova were injected on the left side of the body, and ova from the unidentified species were injected on the right. Two turtles (group C) were given injections of various preparations of tumor at various body sites to test the hypotheses that tumors can be transplanted and that they contain an infectious etiologic agent: 1) double freeze-thawed tumor homogenate was injected into the upper right eyelid, 2) unfrozen tumor homogenate was injected into the upper left eyelid, 3) tumor dermal plugs were implanted into both foreflippers, 4) whole tumor plugs were implanted in both rear flippers, and 5) tumor homogenate was rubbed into scarified neck skin. One turtle (group D) was kept as a control animal with no exposure other than via filtered natural sea water. Turtles were observed daily at feeding and were given careful examinations once a week for inflammation or tumor growth at inoculation sites. Water temperature and salinity were also recorded weekly. Tumors that developed were biopsied and examined by light microscopy and submitted to the Armed Forces Institute of Pathology for examination by transmission electron microscopy.

RESULTS

Immunoglobulin Isolation and Immunoglobulin Specific Mabs

Proteins were eluted from the DEAE column in two peaks corresponding to 0.125 M NaCl and 0.25 M NaCl (fig. 1). Examination of selected fractions by reducing SDS-PAGE revealed that protein from the 0.125 M NaCl peak had three components: 23 Kd, 38 Kd, and 65 Kd (putative light, 5.7S heavy and 7S heavy chains, respectively). Protein in the 0.25 M peak appeared to have two major components: a 23 Kd and a 70 Kd band suggestive of Ig light chain and IgM heavy chain, respectively.

Proteins separated on the Sephacryl S-300 column were eluted in two major peaks: a small early peak containing IgM and a large late peak containing a mixture of 5.7S and 7S IgG (fig. 2). Fractions were examined using SDS-PAGE and those of similar protein composition were pooled. The resulting fraction pools were designated IgM-rich, 5.7S-rich, and 7S-rich and were used for ELISA screening.

A very small amount of sea turtle protein (< 100 ug) was purified by immunoaffinity chromatography from globulin preparation. This material was examined by reducing SDS-PAGE and found to contain major components approximately 65 Kd, 38 Kd, and 23 Kd in size again correlating with 7S and 5.7S heavy chains and light chain.

Hybridoma screening by ELISA and western blotting yielded 28 mass cultures of interest which were retained for further study. Several of these have been cloned and production of

purified monoclonal antibodies initiated. Table 1 gives the specificities and isotypes of these mabs.

The specificity of these mabs is based on their reaction with proteins of the appropriate physical and chemical properties. However, the specificity of these mabs for functional immunoglobulin, i.e., antigen specific antibodies must be determined. Figure 3 shows an example of how one monoclonal, HL 857 (antiturtle 7S IgY) was used to detect a rising antigen specific antibody response in two turtles immunized with DNP-BSA. Figure 4 shows that the response is hapten (DNP) specific because the reaction can be inhibited by soluble DNP. Thus, this mab recognizes a 65 Kd turtle protein that increases in response to immunization and binds specifically to hapten, i.e., a 7S IgY antibody.

Morphological and Cytochemical Characterization of Blood Cells

Green turtle smears examined in this study contained erythrocytes, thrombocytes, monocytes, heterophils, and low numbers of lymphocytes. Eosinophils were rare, and basophils were not observed in our samples. Mature red cells of the green turtle were typically oval with oval or irregularly round nuclei which contained dense, dark-staining chromatin. Immature red cells tended to be more rounded and the cytoplasm was more basophilic. Nuclei tended to be larger with lighter staining clumped chromatin. Punctate basophilic intracytoplasmic bodies were common within red cells. These bodies were PAS positive. Similar bodies have been described for the desert tortoise and have been shown to be remnants of degenerated organelles (Alleman et al., 1992).

In contrast to the platelets of mammals, reptilian thrombocytes are large and nucleated. Thrombocytes are difficult to differentiate from lymphocytes on morphologically. Putative thrombocytes were recognized by their clumping on blood films. Thrombocytes contained a round nucleus containing dense, dark-staining chromatin and scant lightly basophilic cytoplasm. In contrast to lymphocytes the cytoplasm of thrombocytes tended to be more angular and often contained small azurophilic granules. Thrombocytes could be distinguished from lymphocytes using PAS. Thrombocytes showed focal positive-staining, whereas lymphocytes were negative.

Lymphocytes resembled lymphocytes of mammals and other reptiles. They contained scant amounts of basophilic cytoplasm surrounding a single-round nucleus containing clumped chromatin. Cytoplasmic borders of lymphocytes were rounder than those of thrombocytes and the nucleus was lighter staining. Lymphocytes stained negative with all cytochemical stains tested, whereas thrombocytes were PAS positive. One turtle lymphocyte was found which exhibited focal cytoplasmic staining with NBE. This

pattern is characteristic of mammalian T-lymphocytes (Manconi et al., 1979; Wulff et al., 1981).

Heterophils contained a single-round nucleus usually eccentrically located within the cytoplasm. The cytoplasm was filled with numerous eosinophilic granules. The cytoplasm of heterophils showed intense diffuse staining with NBE, CAE, and acid phosphatase. This staining pattern was unique. Eosinophils were NBE negative. In contrast to desert tortoise peripheral blood, green turtles have few, if any, circulating basophils.

Monocytes resembled mammalian monocytes. They contained a single, large, irregular nucleus and a moderate amount of basophilic cytoplasm. The cytoplasm was often vacuolated. Monocytes showed trace-diffuse-cytoplasmic staining with PAS and NBE.

Mabs to Turtle Blood Cells

A single fusion was performed and screened. Screening of the supernatants produced 24 that appeared to react with one or more blood cell populations. Most of these hybridomas remain to be cloned and the cell specificities characterized fully. Also, the target-surface antigens must be identified. Table 1 shows the preliminary characterization of three of these monoclonal antibodies. One hybridoma that appears to be specific for heterophils has been cloned and is presented as an example of the analysis required. Figure 5 shows flow cytometry analysis of green turtle blood leukocytes using light scatter properties (see Shapiro, 1988). Three subpopulations of cells can be recognized on forward- and side-scatter properties alone. These properties roughly correlate with cell size and cytoplasmic granularity, respectively. Figure 6 compares the fluorescence intensity of each cell subpopulation stained with mab HL 879 (MC 137.12 3B2) with their matched controls stained with culture media alone. It is clear that a specific population of cells (R3) is positively stained with this mab. Magnetic beads sensitized with this mab selectively bind to heterophils (confirmed by morphology and cytochemical staining). Overall, the results are very encouraging. With these reagents we can enrich leukocyte preparations for the remaining cell types by removing those cells for which we already have monoclonals, thereby increasing our chances of creating reagents for rarer cell types.

Mabs to GTFP-associated Antigens

One animal was fused and the resulting hybridomas screened. It was not possible to quantify the amount of protein in the Triton X-114 preparation because of detergent interference with protein assays. Blot screening against these crude preparations revealed eight potential hybridomas reactive with tumor. Western

blots were negative, however. Further screening of these mabs is needed.

Green Turtle Antibodies to GTFP Associated Antigens

Results of preliminary indirect testing for tumor-specific turtle antibodies on crude tumor extracts were equivocal. Two-out of 8-tumor bearing animals seemed to have antibodies to unique protein bands, but the results were not consistent. Given the crude nature of the antigen preparation, these results may reflect nonspecific antibody binding or antibodies to contaminants such as bacteria, fungi, parasite eggs, etc. We are in the process of developing fibropapilloma cell lines for testing which should be free of these problems.

Disease Transmission

Over the 11-month observation period reported here no tumors developed in any of the spirorchid egg-treated, water/contact-exposed, or control animals. Some of the injection sites showed mild local swelling that eventually resolved, and was attributed to inflammation.

Both of the tumor-treated turtles (group B) developed fibropapillomas in the right upper eyelid, the site of freeze-thawed homogenate inoculation. Tumors did not develop at any other sites.

Tumors were first clearly recognized as raised cutaneous swelling in one turtle about 4 months after inoculation. The second turtle showed tumor development about 1 month later. Once initiated, growth (measured as increase in tumor diameter) was exponential (fig. 7). The onset of rapid tumor growth was associated with the seasonal increase in water temperature. Tumors were allowed to reach maximum diameters of 2.5 and 6 cm respectively, before they were debulked in September 1992. This was done to preserve the eye on the affected side. Debridement was repeated in March 1993. At maximum size, the tumors were round and relatively smooth. The surface epithelium became ulcerated secondary to repeated trauma against the tank walls.

Histologically, the earliest tumor resembled benign granulation tissue. Later biopsies demonstrate a greatly increased fibrous component with little epidermal involvement other than moderate hyperkeratosis. No viral inclusions or trematode ova were found in the sections examined. Transmission-electron-microscopic examination failed to identify any virus-like particles within the sample.

DISCUSSION

Mabs Against Turtle Immunoglobulins

Sea turtles have three major classes of immunoglobulins: a 17 S IgM, a 7S IgY, and a 5.7S IgY (Benedict and Pollard, 1972 and 1977). As in mammals, IgM is believed to be produced early in an immune response. In addition, IgM may be the primary immunoglobulin that is secreted onto mucosal surfaces (Portis and Coe, 1975). IgY 7S is believed to function as a serum immunoglobulin like mammalian IgG. The role of 5.7S IgY is unclear. Work by Benedict and Pollard (1977) suggests that this is a chronic immune response globulin that appears only after prolonged delay. The ability to detect changes in antigen-specific levels of these three classes may help to establish the chronicity of antigen exposure in a population.

Mabs to green turtle immunoglobulins are critical reagents for characterizing and quantifying the humoral-immune response of these sea turtles to fibropapilloma-associated antigens. They are key components of serodiagnostic tests for detecting exposure to various antigens. We have now produced monoclonal antibodies with putative specificity for green turtle immunoglobulin light chain and each of the known immunoglobulin heavy chain classes. These immunoglobulin classes were isolated based on their physicochemical properties (charge, solubility, size) and relatively pure fractions of each immunoglobulin class were used for immunization and screening.

Initial selection of hybridomas was based on their propensities for binding to turtle proteins with the appropriate physicochemical properties. Experiments are underway to validate monoclonal antibody specificities and to evaluate their performance in various immunoassays. Using captive turtles that were immunized with specific antigens such as DNP-BSA demonstrates that our monoclonal reagents can detect antigen-specific turtle antibodies. The competitive inhibition ELISA assay lends further proof that the proteins detected by our mabs are antigen-specific functional antibodies.

Mabs Against Turtle Blood Cell Types

Methods to isolate and identify various blood cell types are needed to make accurate differential counts, to purify cell types for function studies, and to identify the effector cells, if any, responsible for host resistance to or recovery from fibropapillomatosis. Toward this goal green turtle blood cell morphology and cytochemical staining characteristics were described and monoclonal antibody development begun. The cytochemical staining pattern of green turtle blood cells was similar to that described for desert tortoises, *Gopherus agassazii* (Alleman et al., 1992). Cytochemical staining proved

helpful in distinguishing thrombocytes from lymphocytes, which are morphologically very similar. Cytochemical staining also was a useful adjunct to morphology in distinguishing eosinophils from heterophils and in recognizing monocytes. Specific cell subclasses such as B-lymphocytes, T_{helper}-lymphocytes, $T_{cytotoxic}$-lymphocytes, NK cells, etc., cannot be differentiated readily by cytochemical staining and, therefore, require immunohistochemical phenotyping using monoclonal antibodies against cell surface determinants. Monoclonal antibodies are important reagents for specific staining of blood cell types and can be used to characterize the nature of an inflammatory infiltrate, quantify cell populations, or purify them for function studies. They are especially important when used in flow cytometry to rapidly quantify specific blood cell subpopulations. One hybridoma fusion experiment has been carried out and analyzed which yielded several cell-type-specific mabs. At least one of these hybridomas has been cloned and characterized thoroughly. Each of the others will also require cloning and further screening to fully characterize them and ensure specificity. Our goal is to develop a battery of monoclonals against those leukocytes that may be important in antiviral and antitumor immunity. This will require additional fusion experiments in future years of this project. All of the mabs developed can be used in sequence to selectively deplete turtle cell preparations of those cell types for which we already have reagents (negative selection), thereby enriching those that lack them, thus making it easier to develop monoclonals against these rarer cell types.

GTFP-associated Antigens

Fibropapilloma cells may contain novel antigens against which an affected host might mount an immune response. If any such tumor-associated antigens can be identified and isolated, they could be used in the development of diagnostic tests and perhaps, even vaccines. Possible GTFP-associated antigens include proteins from the putative infectious etiologic agent (virus), viral proteins expressed only within the host cell, oncogene products, normal proteins produced in abnormal amounts or at an inappropriate time. A serum-based diagnostic test for exposure to the fibropapillomatosis agent or development of subclinical tumors would rely on shedding of tumor-associated antigens into the plasma and/or development of an anti-GTFP immune response with circulating GTFP-specific antibodies.

The approach to studying GTFP antigens has been two-pronged. First, we attempted to develop GTFP-specific mabs. Second, we began searching for evidence of antibodies directed against tumor tissue in the serum of turtles with fibropapillomatosis.

One fusion yielding eight mabs with potential tumor-specific reactivity has been performed. These mabs have not been fully

characterized yet. They appear to react with TX-114 tumor extracts in dot blots but not in western blots and their reactivity to normal skin extracts remains to be tested. Full characterization will require better antigen preparations produced from tumor and normal skin or from cultured cell lines.

The search for tumor-specific turtle antibodies against fibropapilloma has only just begun because all reagents have been still under development. However, we undertook some preliminary experiments using crude tumor extracts and sera from tumor-free and tumor-bearing turtles. Some sera from tumor-bearing turtles appeared to react with a unique protein band but the nature of this band has not been characterized. This pattern of results is not unexpected because the humoral immune response to tumor-associated antigens may vary widely among individuals depending on their health status, stage of the disease, and previous disease history. False positive reactivity is a major problem using crude tumor preparations because the preparation may contain antigens from passenger microbes and parasites having nothing to do with the disease. Definitive experiments will require more highly purified antigen preparations either from proven GTFP cell lines or an identified etiologic agent. If GTFP is caused by a virus, then we expect to be successful in developing assays to detect the turtle humoral immune response to viral antigens (see Beiss et al., 1987; Viac, et al., 1990). In addition, Barthold and Olson (1974 and 1978) were able to detect the presence of tumor-associated (non virion) antigen-specific antibodies in sera from cattle and horses affected with fibropapillomatosis using tumor-derived fibroblasts as target cells. We hope to be able to conduct similar experiments in the near future and are developing GTFP cell lines for this purpose.

Transmission Study

Transmission studies are needed to rule in or out an infectious etiology and to begin to understand the effective routes of transmission. In addition, it is critical to be able to induce the disease in naive recipients to study progression and regression and the host-immune response to the tumor. Finally, being able to transplant tumor cells is important in demonstrating the neoplastic nature of the disease (vs. aberrant wound response) and verifying the tumorigenic phenotype of the cells.

Preliminary transmission experiments were conducted by Jacobson, Sundberg, Moretti, and Brown (unpubl.) in 1990 using adult captive-reared green turtle recipients. This experiment produced negative results after 1 year of monitoring. The experiments described in this report yielded some exciting preliminary results that demonstrate, for the first time, that this tumor can be experimentally transmitted from one turtle to another. The pattern of results is consistent with a viral

etiology because freeze-thawed tumor homogenate was the only material that elicited tumor growth. This treatment should have lysed tumor cells and released virus particles or viral DNA/RNA. However, these results are insufficient to clearly implicate an infectious viral agent as the etiology of GTFP because the homogenate was not filtered to preclude transfer of intact viable cells or bacteria. Furthermore, these data are unable to elucidate the mode of transmission of this disease in nature. The advantages of this breakthrough are (1) that tumor material of defined origin and age eventually will become available for studies of GTFP progression, host susceptibility, host-immune response, and anti-GTFP therapy, and (2) GTFP cell lines maintained for in vitro studies can be checked for the tumorigenic phenotype in vivo.

The fact that trematode eggs did not lead to tumor development in this study, suggests that eggs do not play a direct role in pathogenesis and that the presence of eggs in tumors may be incidental (see Smith and Coates, 1939; Lauckner, 1985). However, this experiment was conducted as a single dose acute exposure which may not mimic the natural condition. Further experiments using various doses and dosing intervals are needed to rule out any involvement of trematode ova in the pathogenesis of this tumor.

Water exposure or contact with affected individuals did not lead to tumor development during the course of this experiment. It may be that these are effective but inefficient routes of transmission in nature, given much longer periods of exposure. It also remains possible that the development of natural GTFP is a complex phenomenon involving multiple factors and biochemical alterations occurring over long time spans.

These preliminary results are encouraging but much further work is needed to determine the parameters for reliable tumor transmission and to elucidate the pathogenic mechanisms of this disease. Because the double freeze-thawed GTFP homogenate appeared to be the effective treatment we have performed a cross-over experiment with the trematode ova treated and water-exposed turtles. Two turtles from each group were treated in September 1992 with either double freeze-thawed GTFP homogenate or filtered freeze-thawed homogenate. The purpose of this experiment is to ask whether cell-free extract (filtered homogenate) can induce tumor growth. If successful, this would point to a filterable agent (virus) as the etiology. Presently, we cannot be sure whether the 100% success rate in the tumor-treated group was due to a virus or transplantation of surviving tumor cells.

We are planning additional experiments with a new group of 20 post-hatchling turtles this year. The experiment will focus on demonstrating whether a filterable agent (virus) can cause the tumors. The preliminary results are encouraging but future

results will require considerable time to collect because of the long latent period for onset of detectable tumors.

CONCLUSION

Overall, a substantial amount of progress has thus far been made in this project toward the stated objectives. Future work will continue development and refinement of immunodiagnostic reagents and will continue to focus on identifying antigens that can serve as markers for GTFP exposure. This will be accomplished by identifying and culturing an infectious etiologic agent or identifying tumor-associated antigens in GTFP tumor cells.

ACKNOWLEDGMENTS

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Table 1.--Catalog of cloned hybridomas producing monoclonal antibodies against green turtle immunoglobulins and blood cells.

Designation	Specificity[a]	Isotype
HL 814	5.7S IgY heavy chain	n.t.
HL 821	IgM heavy chain	IgG_1
HL 846	IgM heavy chain	IgG_1
HL 848	Light chain	IgG_1
HL 857	7S IgY heavy chain	IgG_{2A}
HL 859	5.7S IgY heavy chain	IgG_1
HL 879	Heterophil	IgG_1
MC137.12 1A5[b]	Erythrocyte	n.t.
MC137.12 4B4[b]	Monocyte	n.t.

[a]Presumptive Ig specificities tested by ELISA and Western blot. Cell specificities confirmed by magnetic bead assay, cytochemical staining, and flow cytometry.
[b]Cloning in progress.

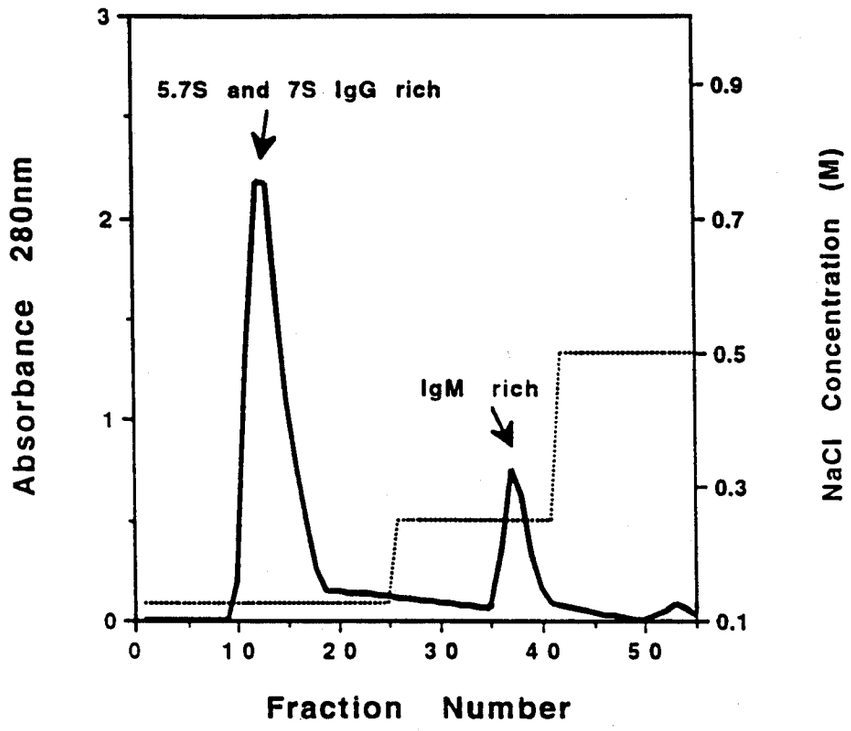

Figure 1. Fractionation of green turtle immunoglobulins by anion exchange chromatography. Protein composition of fractions was examined with SDS-PAGE. The dotted line represents the steps of increasing salt concentration (0.125, 0.25, and 0.5 M NaCI respectively).

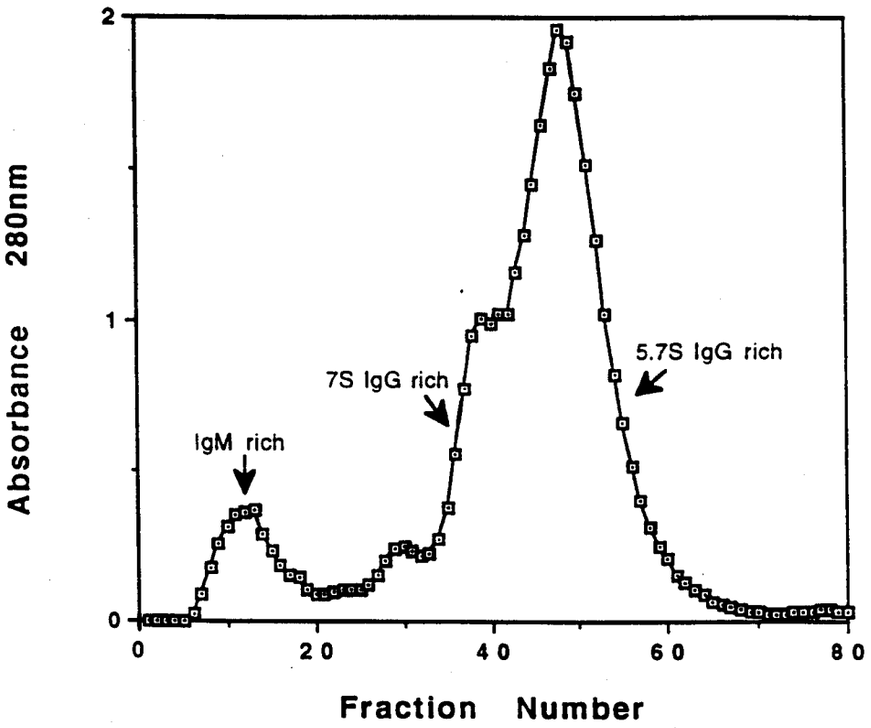

Figure 2. Fractionation of green turtle immunoglobulins by size exclusion chromatography. The protein composition of fractions was examined with SDS-PAGE.

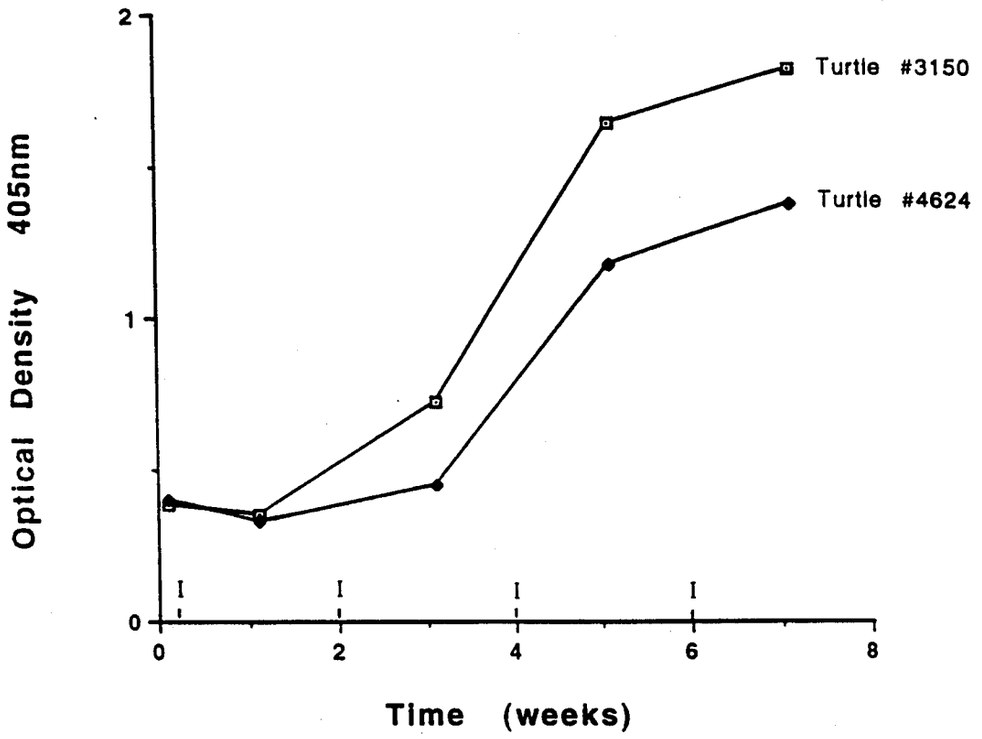

Figure 3. Green turtle 7S IgG anti-DNP/BSA response. Two turtles were immunized (I) at 2-week intervals with 250 ug DNP/BSA in RIBI's adjuvant. Plasma samples were collected before immunization and at 2-week intervals between inoculations. Turtle sera were assayed at 1:40 dilution. ELISA plates were coated with 50 ul DNP/BSA at 1.25 ug/ml coating concentration. Mab HL 857 anti-turtle 7S IgY was used as primary antibody. Optical density was read after 60 minutes incubation with substrate.

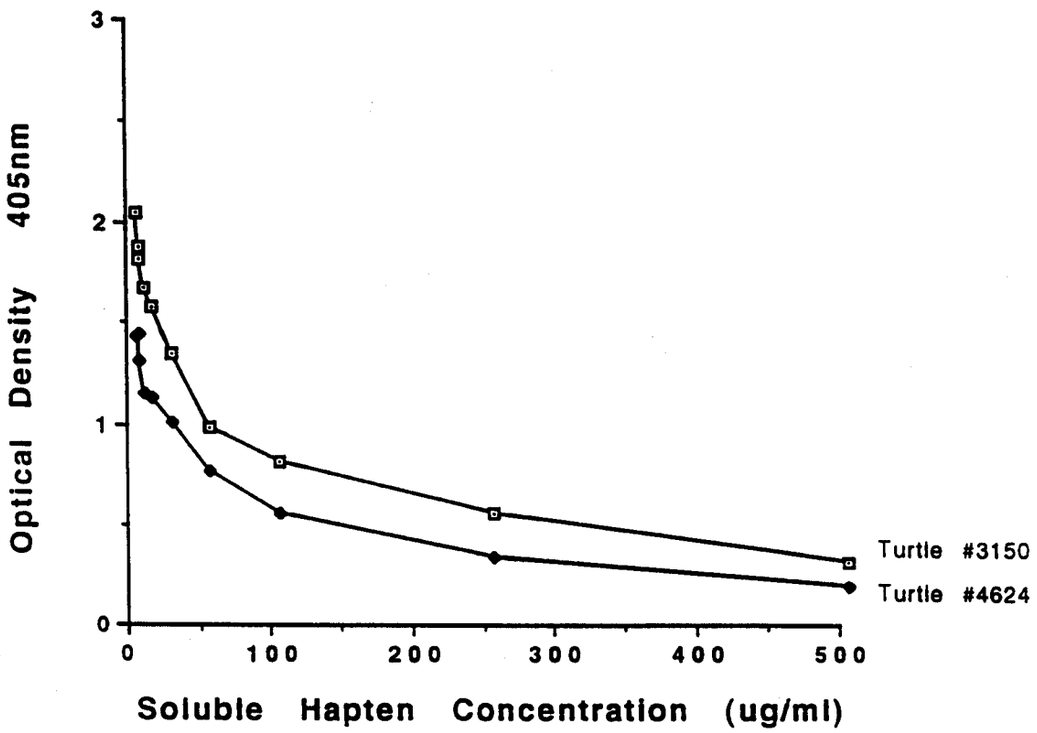

Figure 4. Inhibition of plasma anti-DNP/BSA reactivity by soluble hapten. Turtle plasma samples (1:40 dilution) taken at the peak 7S IgY anti-DNP/BSA response (7 weeks) were incubated overnight with 0, 1, 2, 5, 10, 25, 50, 100, 250, or 500 ug/ml DNP-glycine. The samples were then examined by ELISA using HL 857 anti-turtle 7S IgY as primary antibody. Optical density was read at 60 minutes incubation with substrate.

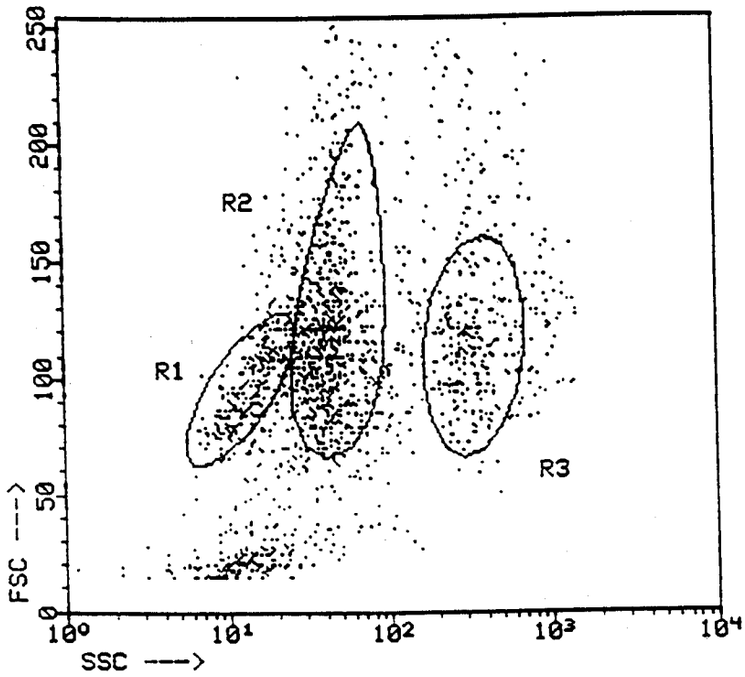

Figure 5. Flow cytometric analysis of green turtle peripheral blood leukocytes by light scatter. A total of 3 x 10^4 cells were counted but 2 x 10^3 cells (dots) were plotted for clarity. Three main blood cell subpopulations R1, R2, and R3 can be recognized by their forward scatter (FSC) and side scatter (SSC) properties.

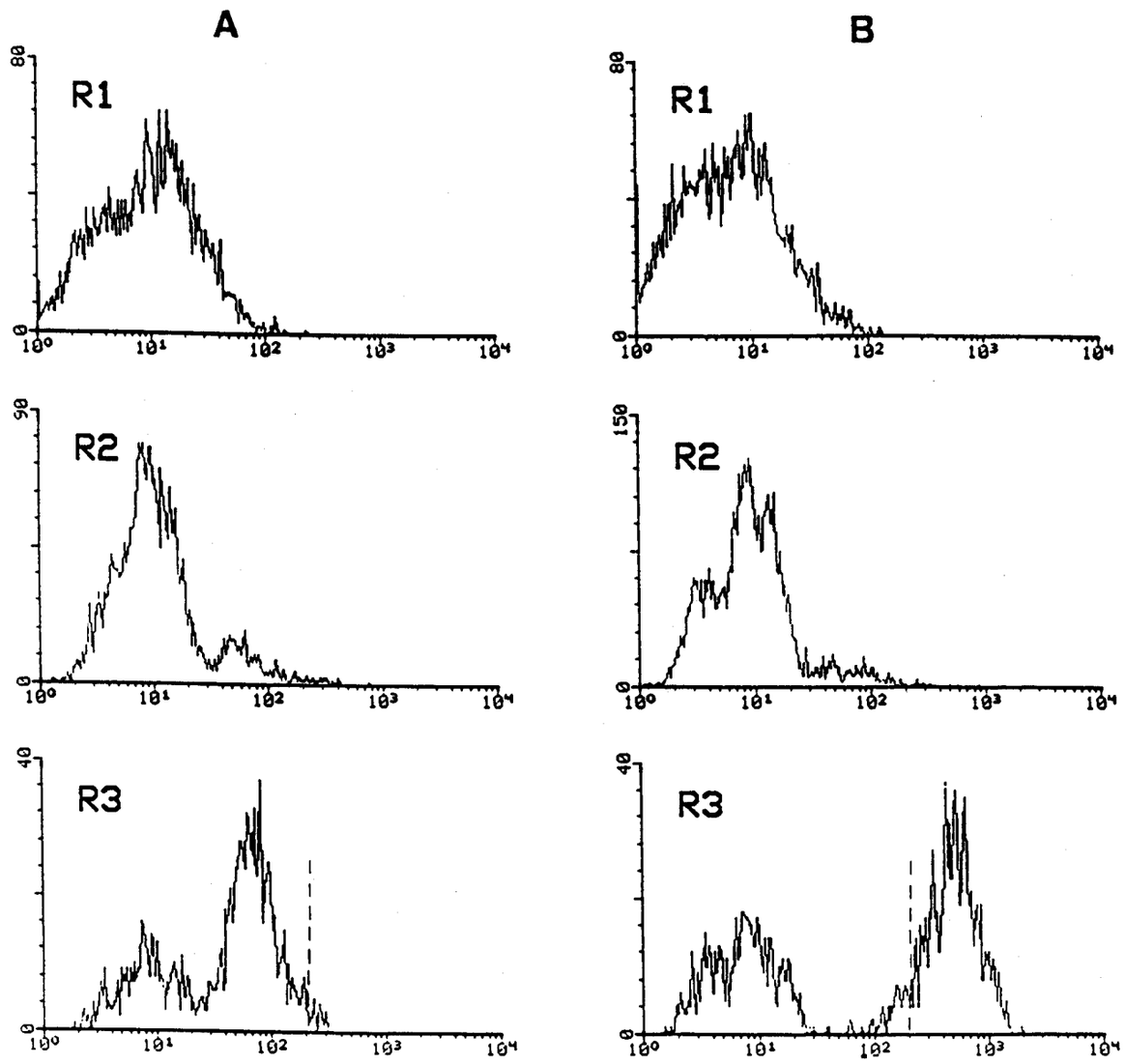

Figure 6. Flow cytometric analysis of green turtle blood cell subpopulations by immunofluorescence. Peripheral blood leukocytes ("buffy coat" cells) were incubated with either **A)** cell culture media (control) or **B)** mab HL 879 culture supernatant (anti-turtle heterophil) followed by FITC labelled Fab' sheep anti-mouse IgG. Subpopulations R1, R2, and R3 were identified by light scatter properties (see fig. 5). Shown are frequency histograms with cell number plotted on the y-axis and fluorescence intensity (log scale) on the x-axis. Only R3 cells show positive staining relative to controls.

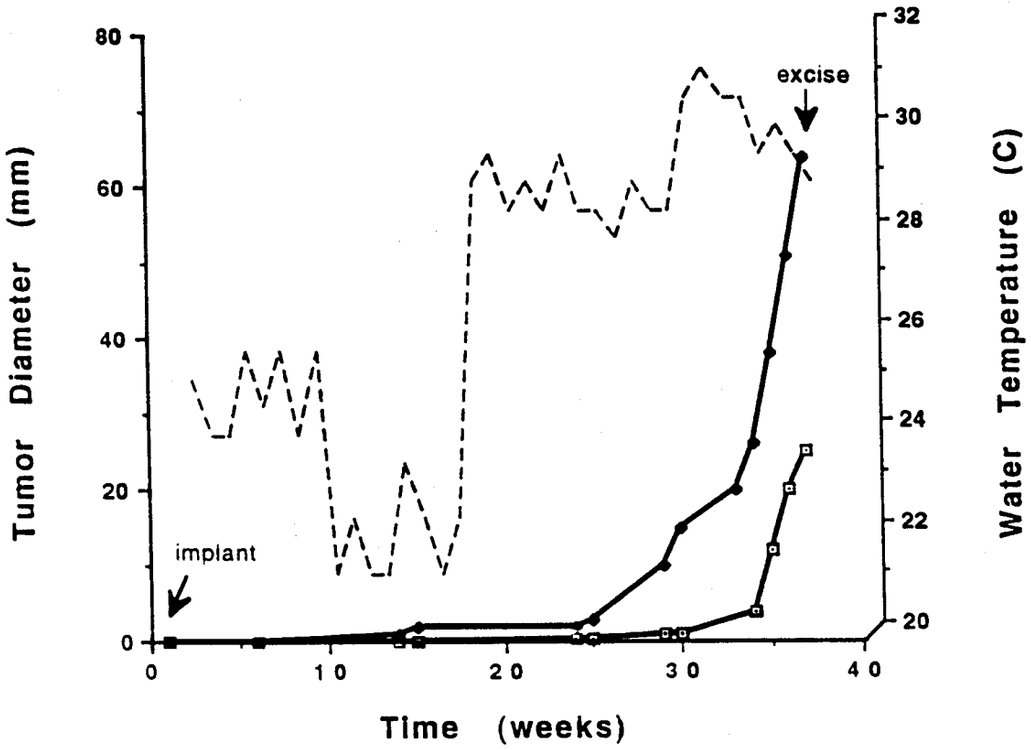

Figure 7. Tumor growth following experimental transmission. Two turtles receiving 2x freeze-thawed GTFP homogenate developed tumors at the site of injection. The solid lines show the increase in tumor diameter over time for both turtles. The dashed line illustrates the weekly fluctuation in water temperatures during the same time period.